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Structure-Function Analysis of SH3 Domains: SH3 Binding Specificity Altered by Single Amino Acid Substitutions

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SH3 domains mediate intracellular protein-protein interactions through the recognition of proline-rich sequence motifs on cellular proteins. Structural analysis of the Src SH3 domain (Src SH3) complexed with proline-rich peptide ligands revealed three binding sites involved in this interaction: two hydrophobic interactions (between aliphatic proline dipeptides in the SH3 ligand and highly conserved aromatic residues on the surface of the SH3 domain), and one salt bridge (between Asp-99 of Src and an Arg three residues upstream of the conserved Pro-X-X-Pro motif in the ligand). We examined the importance of the arginine binding site of SH3 domains by comparing the binding properties of wild-type Src SH3 and Abl SH3 with those of a Src SH3 mutant containing a mutated arginine binding site (D99N) and Abl SH3 mutant constructs engineered to contain an arginine binding site (T98D and T98D/F91Y). We found that the D99N mutation diminished binding to most Src SH3-binding proteins in whole cell extracts; however, there was only a moderate reduction in binding to a small subset of Src SH3-binding proteins (including the Src substrate p68). p68 was shown to contain two Arg-containing Asp-99-dependent binding sites and one Asp-99-independent binding site which lacks an Arg. Moreover, substitution of Asp for Thr-98 in Abl SH3 changed the binding specificity of this domain and conferred the ability to recognize Arg-containing ligands. These results indicate that Asp-99 is important for Src SH3 binding specificity and that Asp-99-dependent binding interactions play a dominant role in Src SH3 recognition of cellular binding proteins, and they suggest the existence of two Src SH3 binding mechanisms, one requiring Asp-99 and the other independent of this residue.

The SH3 (Src homology 3) domain is a small noncatalytic domain of 50 to 60 amino acids that has been identified in many intracellular signaling proteins, including Src and Abl family protein tyrosine kinases (13, 14). This domain mediates protein-protein interactions that are important for coupling of intracellular signaling pathways, regulation of catalytic activity of proteins, recruitment of substrates to enzymes, and localization of proteins to a specific subcellular compartment (4, 9). Numerous studies have shown that SH3 domains recognize specific cellular proteins by interactions with short contiguous peptide sequences rich in proline residues (3, 4, 15).

Several strategies have been used to identify high-affinity SH3-binding peptides. Combinatorial peptide libraries revealed two classes of Src and phosphatidylinositol 3′-kinase (PI3K) SH3 ligands. Class I peptides have an N-terminal Arg with the consensus sequence RXLPPZP (Z represents Leu for the Src SH3 domain [Src SH3] and Arg for PI3K SH3), whereas class II peptides have a C-terminal Arg with the consensus sequence XPPLPXR. Phage display libraries have also been used to define the minimal core recognition motifs for Src, Fyn, Lyn, Abl, and PI3K domains as well as the additional flanking sequences preferred by each individual SH3 domain (1, 17, 18, 21). It has been shown that while the SH3 domains of Src, Fyn, Lyn, and PI3K all select a core recognition motif, RPLPPLP, with some preference for distinct residues in the regions flanking the core motif, Abl SH3 prefers the sequence PPPYPPPP(I/V)P (the underlined sequences indicate the highly conserved PXXP motif present in all SH3 ligands).

Analysis of the structures of Src, Fyn, PI3K, and Abl SH3 domains complexed with their peptide ligands revealed that the ligands adopt a left-handed type II polyproline (PPII) helix that intercalates into the binding pockets formed on the surface of the SH3 domains (6, 10, 26). For example, the PPII helix formed by a class I Src SH3 ligand, $R_{-3}A_{-2}L_{-1}P_0P_1L_2P_3R_4Y_3$, interacts with three binding pockets on Src SH3 (Fig. 1A). The first binding site (formed by Tyr-90 and Tyr-136 of Src) interacts with P_3 and L_2 of the ligand. The second one is formed by the highly conserved aromatic residues (Tyr-92, Trp-118, Pro-133, and Tyr-136) that make extensive hydrophobic contacts with L_{-1} and P_0 of the ligand. The third one (positioned between Trp-118 and Asp-99) is an arginine binding site in which Asp-99 appears to interact with Arg_{-3} via a salt bridge. Asp-99 is also implicated in ligand selection, since all class I Src SH3 ligands selected from peptide and phage display libraries contain an Arg N terminal to the Pro-X-X-Pro motif. Furthermore, all of the SH3 domains that contain an acidic residue analogous to Src Asp-99 (including PI3K, Fyn, and Lyn) preferentially select sequences with an Arg at the same position in class I ligands (17). The Src SH3 and PI3K SH3 also selected ligands with an Arg two residues C terminal to the X-Pro-Pro-X-Pro motif in class II ligands (X-Pro-Pro-X-Pro-X-Arg). The C-terminal Arg of a Src SH3

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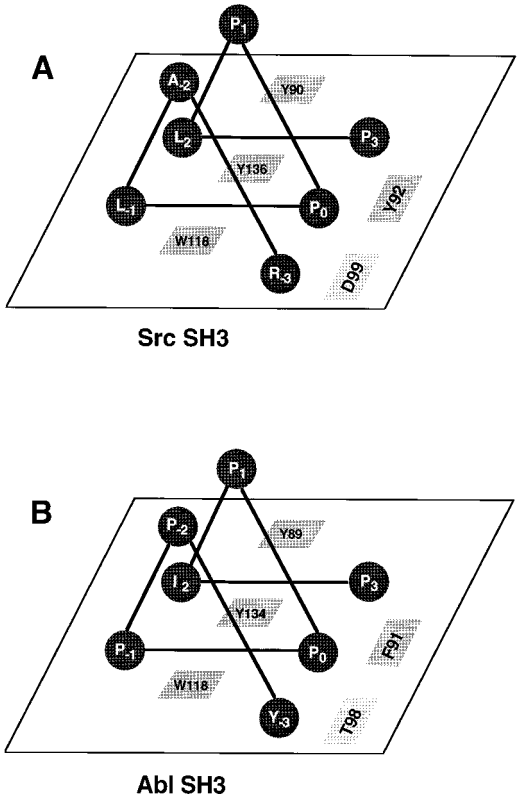


FIG. 1. Schematic diagrams illustrating PPII helices formed by a Src SH3 ligand (A) and an Abl SH3 ligand (B) at the binding site of Src SH3 and Abl SH3. Panel A is modified slightly from a figure that appeared in reference 6. Panel B is based on panel A and the structural analysis of Abl SH3 complexed with a 3BP-1 peptide (10). An incomplete Abl SH3 ligand (YPPPPIP) selected from the phage display library (17) is used in panel B, since the additional amino acids (three prolines) N terminal to the Tyr at position −3 may adopt an extended conformation other than the PPII helix conformation as suggested by Musacchio et al. (10).

ligand also interacts with Src Asp-99, yet the ligand binds to the SH3 domain in a reverse orientation (6).

The majority of known SH3 domains contain an acidic amino acid residue at the position analogous to Asp-99 of Src. One exception is Abl SH3, which contains a Thr at this position (Fig. 1B). This difference may provide a plausible explanation for the Abl SH3 selection of a Tyr instead of an Arg at the −3 position in peptides from phage display libraries (17) and a Met in the −3 position of proteins selected from λgt11 cDNA libraries (3).

Although all of the Src SH3 peptide ligands selected from either phage display or peptide libraries contain an Arg_{-3} that appears to interact with Src Asp-99 via a salt bridge, some of the natural SH3 recognition motifs identified in cellular proteins that bind to Src SH3 lack this analogous arginine for such an interaction (e.g., paxillin, Shc, and AFAP). To investigate the potential importance of Src Asp-99 in binding to cellular proteins and peptide ligands, we substituted Src Asp-99 with an Asn (D99N) and compared the binding properties of the mutant and wild-type SH3 domains. We also analyzed two Abl SH3 mutants that were engineered to contain an Asp substitution for Thr-98, the residue analogous to Src Asp-99. These studies indicated that while Asp-99 is required for binding to most cellular SH3-binding proteins and for selection of peptides containing an Arg at position −3, it is not critical for selection of peptide ligands or cellular proteins that lack an Arg in this position. In addition, the substitution of Asp in the analogous position Abl SH3 altered the binding properties of this protein and conferred binding activity to ligands containing an Arg at the −3 position. These studies highlight the critical role the Asp-99 position in the binding selectivity of Src SH3 and provide the first example of protein engineering to shift the binding specificity of an SH3 domain.

MATERIALS AND METHODS

Generation of recombinant pGEX-2T plasmids and GST fusion proteins. The sequence encoding the SH3 domain of chicken c-Src (amino acids 81 to 147) or a mutant variant (D99N) was cloned into the pGEX-2T vector (20) as described before (6, 24). pGEX-2T-Abl SH3 encoding the SH3 domain of the murine type IV c-Abl (amino acids 84 to 138) was kindly provided by D. Baltimore (3). The two Abl SH3 mutants (F91Y/T98D and T98D) were generated by using the transformer site-directed mutagenesis kit (Clontech, Palo Alto, Calif.) according to the manufacturer's instructions. p68 and its deletion constructs were cloned into myc-Bluescript KS+ as described previously (16). The recombinant and mutated plasmids were subjected to double-stranded DNA sequencing to verify sequence integrity. Glutathione *S*-transferase (GST) and GST-SH3 fusion proteins were generated as previously described (24).

Affinity binding assay. The affinity binding assay was performed as described before (23, 24). Briefly, BALB/c 3T3 cells were labeled overnight with 50 μCi of [^{35}S]methionine and then lysed with radioimmunoprecipitation assay (RIPA) buffer (158 mM NaCl, 5 mM EDTA, 10 mM Tris [pH 7.2], 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 1% Triton X-100). Following clarification at 28,000 × *g*, supernatants were incubated for 4 h at 4°C with 50 μl of glutathione-agarose (1:1 slurry) bound with 50 μg of GST or GST-SH3 fusion proteins. After washing, the binding proteins were eluted with Laemmli sample buffer and fractionated on an SDS–8% polyacrylamide gel. The gel was soaked in 1 M sodium salicylate for 1 h, dried, and exposed to film at −70°C.

Western blotting (immunoblotting). v-Src-transformed BALB/c 3T3 (SRD 3T3) cells were lysed with either RIPA buffer or Nonidet P-40 (NP-40) buffer (100 mM NaCl, 20 mM Tris [pH 7.5], 1% NP-40, 10% glycerol), and the affinity binding assays were performed as described above. The samples were separated by electrophoresis on 10% polyacrylamide gels and transferred to nitrocellulose. The blots were incubated in blocking buffer (5% crystallized bovine serum albumin, 170 mM NaCl, 0.2% NP-40, 50 mM Tris [pH 7.5]) for at least 0.5 h at room temperature. The filters were probed with the phosphotyrosine monoclonal antibody 4G10 (kindly provided by T. Roberts, Dana-Farber Cancer Institute), heterogeneous nuclear ribonucleoprotein (hnRNP) K monoclonal antibody 3C2 (8), a p68 polyclonal antibody (Santa Cruz Biotechnology), or Myc monoclonal antibody 9E10 (American Type Culture Collection, Rockville, Md.) followed by a horseradish peroxidase-coupled secondary antibody (Bio-Rad). Immunoreactivity was detected by enhanced chemiluminescence (Amersham).

Tryptophan fluorescence spectroscopy. Four peptides were used in this assay: RPL (APARPLPPLPGGK), RPP (APARPPPPLPGGK), YPP (APAYPPPPLP GGK), and YPL (APAYPLPPLPGGK). GGK was added to the carboxy terminus of each of the peptides (the C-terminal lysine allows immobilization of the peptide to solid phase via standard hydroxysuccimide chemistry for BIAcore analysis, and the pair of glycines provide a spacer to minimize steric interference from the solid support). Fluorescence measurements were carried out with a Perkin-Elmer LS50B luminescence spectrophotometer at room temperature. The excitation wavelength used in most of the experiments was 280 nm (5-nm slit), and the emission wavelength was 320 or 330 nm (5-nm slit). Fluorescence intensity measurements for peptides YPP and YPL were performed with an excitation wavelength of 295 nm (5-nm slit) to minimize tyrosine fluorescence. In a typical assay, aliquots of peptide solution were added to 3.0 ml of 0.5 μM GST-SH3 solution in phosphate-buffered saline (pH 7.4) (GIBCO), and the mixture was stirred in a cuvette for at least 5 min prior to analysis with the spectrophotometer. The peptide solution was usually added until no significant SH3-dependent changes in fluorescence intensity were observed. After background subtraction and volume correction, the dissociation constant (K_d) was calculated as described below (2).

Assuming a one-to-one complex between an SH3 domain and a peptide ligand, the F_{max} can be first extrapolated by using nonlinear regression (equation 1), and the K_d value between an SH3 domain and its ligand can then be calculated by Scatchard analysis, using equation 2:

$$F = F_0 + (F_{max} - F_0)([L]/([L] + K_d)) \quad (1)$$

$$(F - F_0)/(F_{max} - F_0) = 1 - K_d\{(F - F_0)/(F_{max} - F_0)\}/[L] \quad (2)$$

F_0 represents the fluorescence intensity of a free SH3 domain, F_{max} is the fluorescence intensity of the SH3 domain saturated with its ligand, and $[L]$ is the concentration of the ligand.

Selection of SH3 ligands from phage display libraries. SH3 peptide ligands were selected from a biased X_6PPIP library (containing six randomized amino acids N terminal to the fixed tetrapeptide PPIP) as previously described (17).

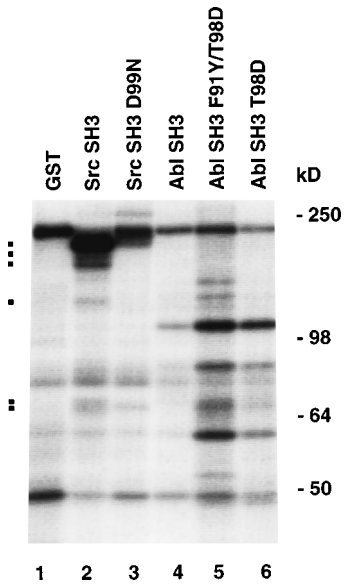


FIG. 2. Binding of cellular proteins to the SH3 domains of Src and Abl and their mutant variants. [^{35}S]methionine-labeled SRD 3T3 cell lysates were precleared by incubation with GST-agarose and then incubated with glutathione-agarose bound with GST (lane 1), GST-Src SH3 (lane 2), GST-Src SH3 D99N (lane 3), GST-Abl SH3 (lane 4), GST-Abl SH3 F91Y/T98D (lane 5), or GST-Abl SH3 T98D (lane 6) as described in Materials and Methods. Bound proteins were eluted with SDS sample buffer, fractionated on an SDS–8% polyacrylamide gel, and visualized by autoradiography. The Src SH3-binding proteins are indicated with squares.

RESULTS

Isolation of SH3-binding proteins in BALB/c 3T3 lysates, using wild-type Src and Abl SH3 and their mutant variants. We previously detected Src SH3-binding proteins in normal BALB/c 3T3 and SRD 3T3 cells by using a GST-Src SH3 fusion protein as an affinity matrix (19, 23, 24). To examine the importance of Src SH3 Asp-99 in mediating binding to these cellular proteins, we performed a similar binding assay using equal amounts of wild-type Src SH3 and the D99N mutant variant of Src SH3 expressed as GST fusion proteins. For comparison, we also analyzed cellular proteins that can bind to wild-type Abl SH3 and the T98D mutant to examine whether the T98D substitution changes the Abl SH3 binding specificity. In addition, since the structural analysis of Src SH3 revealed the existence of a hydrogen bond between Tyr-92 and Asp-99 in Src SH3, we also substituted Abl Phe-91 (structurally equivalent to Src Tyr-92) with a Tyr and included the double mutant (Abl SH3 F91Y/T98D) in the same binding assay.

Figure 2 shows the proteins from [^{35}S]methionine-labeled BALB/c 3T3 cell lysates that were isolated by using the GST-SH3 fusion proteins and glutathione-agarose. A number of proteins were affinity purified by using GST-Src SH3 (e.g., 200, 180, 160, 140, 70, and 68 kDa) (lane 2). While D99N greatly reduced or abolished binding of most of these proteins to Src SH3 (e.g., 200, 180, 160, 140, and 70 kDa), binding of a 68-kDa protein was not affected by this mutation (lane 3). The wild-type Abl SH3 (lane 4) specifically bound to a 120-kDa protein that was distinct from Src SH3-binding proteins, and two Abl SH3 mutant variants (T98D and F91Y/T98D) exhibited higher affinity for this protein (lanes 5 and 6). These two Abl SH3 mutants also bound to several proteins (e.g., 85 and 60 kDa) that were not detected by using wild-type Abl SH3. In addition, the double mutant F91Y/T98D appeared to bind to two proteins (140 and 160 kDa) that were not isolated by the T98D mutant. Together, these results demonstrate that Asp-99 of Src is important for mediating binding of most cellular proteins to Src SH3 and that the T98D substitution in Abl SH3 can modify the binding specificity of Abl SH3.

Tyrosine phosphorylation of SH3-binding proteins. We previously showed that Src SH3 specifically interacted with several tyrosine-phosphorylated proteins from SRD 3T3 cells. Figure 3 shows an antiphosphotyrosine immunoblot of proteins affinity purified from SRD 3T3 cells by using glutathione-agarose bound with equal amounts of either GST (lane 1) or GST-SH3 fusion proteins (lanes 2 to 6). Consistent with previous findings, Src SH3 (lane 2) bound to several tyrosine-phosphorylated proteins with apparent molecular masses of 68, 130, and 160 kDa. While D99N mutation abolished binding of 130- and 160-kDa tyrosine-phosphorylated proteins to Src SH3 (lane 3), this mutation only moderately reduced binding of a 68-kDa tyrosine-phosphorylated protein to this domain. Although no apparent binding of any of these tyrosine-phosphorylated proteins to GST-Abl SH3 was observed (lane 4), a 68-kDa tyrosine-phosphorylated protein was found to bind to both GST-Abl SH3 F91Y/T98D and GST-Abl SH3 T98D (lanes 5 and 6). These results demonstrate that Asp-99 of Src plays a role in binding of Src SH3 to some tyrosine-phosphorylated proteins and that the T98D substitution in Abl SH3 allowed this domain to bind to a 68-kDa tyrosine-phosphorylated Src substrate.

Comparison of binding of p68 and hnRNP K to the wild-type and mutant SH3 domains. We previously identified four cellular proteins that can bind to Src SH3: p68 (a target of Src during mitosis), hnRNP K (a pre-mRNA-binding protein), Shc (a signaling protein that couples membrane tyrosine kinases with Ras), and paxillin (a cytoskeleton protein found in focal adhesions) (19, 23, 24). Since p68 is one of the 68-kDa tyrosine-phosphorylated proteins associated with Src SH3 (Fig. 3, lane 2) and the SH3 binding sites in p68 and hnRNP K (a p68-related protein) have been characterized (16), we directly examined the ability of p68 and hnRNP K to interact with the wild-type Src SH3 and its D99N mutant. We also included two Abl SH3 mutants (T98D and F91Y/T98D) in the binding assay to examine whether either of the two amino acid substitutions would enable Abl SH3 to bind to p68 or hnRNP K.

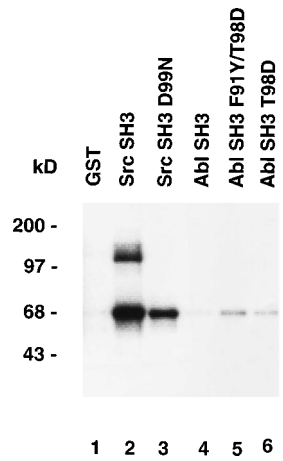


FIG. 3. Tyrosine phosphorylation of the SH3-binding proteins. SRD 3T3 cells were lysed with RIPA buffer and incubated with glutathione-agarose bound with GST (lane 1), GST-Src SH3 (lane 2), GST-Src SH3 D99N (lane 3), GST-Abl SH3 (lane 4), GST-Abl SH3 F91Y/T98D (lane 5), or GST-Abl SH3 T98D (lane 6) as described in Materials and Methods. The bound proteins were eluted with SDS sample buffer, fractionated on an 8% polyacrylamide gel, transferred to nitrocellulose, and probed with the phosphotyrosine monoclonal antibody 4G10.

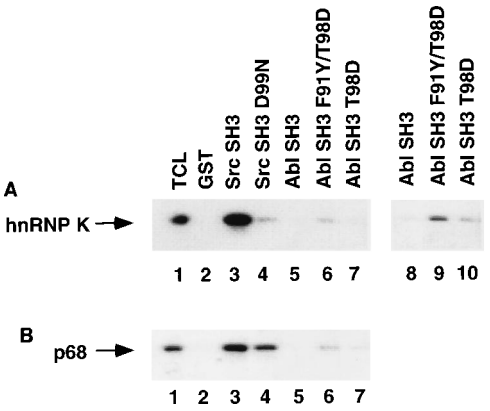


FIG. 4. Comparison of binding of hnRNP K and p68 to the wild-type and mutant SH3 domains. (A) SRD 3T3 cells were lysed with NP-40 buffer and incubated with glutathione-agarose bound with GST (lane 2), GST-Src SH3 (lane 3), GST-Src SH3 D99N (lane 4), GST-Abl SH3 (lane 5 and longer-exposure lane 8), GST-Abl SH3 F91Y/T98D (lane 6 and longer-exposure lane 9), or GST-Abl SH3 T98D (lane 7 and longer-exposure lane 10). The bound proteins were eluted with SDS sample buffer, fractionated on an 8% polyacrylamide gel along with 75 μg of total cellular lysates (TCL) of SRD 3T3 (lane 1), transferred to nitrocellulose, and probed with a mouse monoclonal antibody against hnRNP K as described in Materials and Methods. (B) The blot shown in panel A was stripped and probed with a rabbit polyclonal antibody against p68 as described in Materials and Methods.

Figure 4A is an hnRNP K immunoblot showing that the D99N mutation in Src SH3 greatly reduced the binding of hnRNP K to Src SH3: only approximately 1/10 of the hnRNP K that bound to wild-type Src SH3 could bind to the Src D99N mutant (lanes 3 and 4). We recently mapped the SH3 binding site in hnRNP K to a 36-amino-acid region containing two proline-rich stretches (RGPPPPP and RNLPLPP). Since both of these two proline-rich stretches contain an Arg_{-3} to potentially interact with Src Asp-99, it is not surprising that binding of hnRNP K to Src SH3 is dependent on Src Asp-99. Interestingly, although no apparent binding of hnRNP K to wild-type Abl SH3 was detected (lanes 5 and 8; lane 8 is a longer exposure of lane 5), a small amount of hnRNP K was found to bind to both Abl SH3 F91Y/T98D (lanes 6 and 9; lane 9 is a longer exposure of lane 6) and Abl SH3 T98D (lanes 7 and 10; lane 10 is a longer exposure of lane 7). These data suggest that binding of hnRNP K to Src SH3 is predominantly dependent on Src Asp-99 and that the T98D substitution in Abl SH3 confers the ability of this domain to bind to hnRNP K.

Interestingly, although the D99N mutation almost completely diminished binding of hnRNP K to Src SH3, the same binding assay (Fig. 4B) revealed that the D99N mutation only moderately reduced the binding of p68 to Src SH3 (approximately 50% reduction; compare lanes 3 and 4). Although no apparent binding of p68 to wild-type Abl SH3 was detected (lane 5), F91Y/T98D and T98D substitutions in Abl SH3 enabled this domain to weakly associate with p68 (lane 6 and 7). Together, the data suggest that the interaction of p68 with Src SH3 is complex and only partially dependent on Asp-99 of Src.

Analysis of SH3 binding sites in p68 with the wild-type and mutant SH3 domains. Using an in vitro binding assay, Richard et al. recently identified three SH3 binding sites in p68: P3 (PPPPPVPRGR), P4 (RGVPPPP), and P5 (PLPPPAP) (as indicated on the left in Fig. 5) and demonstrated that the P5 site mediates most of SH3 binding while the P4 and P3 sites mediate some degree of binding (16). Since two of the SH3 binding sites (P3 and P4) in p68 contain arginines and the third (P5) lacks an arginine, we used epitope-tagged p68 deletion mutants containing either P3, P4, or P5 to examine the role of arginines in Src SH3-p68 binding.

HeLa cells expressing the wild-type epitope-tagged p68 (p68-Myc) or its deletion mutants were lysed with Triton buffer, and the lysates were incubated with GST-SH3 fusion proteins and glutathione-agarose. Bound proteins were transferred to nitrocellulose and detected with antibody 9E10, which recognizes the Myc epitope tag. Consistent with the results shown in Fig. 4B, p68-Myc bound well to the wild-type Src SH3, and the D99N mutation in Src SH3 resulted in approximately 50% of p68 binding (Fig. 5A, lanes 3 and 4). While no apparent binding of p68-Myc to GST-Abl SH3 was detected (Fig. 5A, lane 5), p68-Myc was found to bind weakly to GST-Abl SH3 T98D

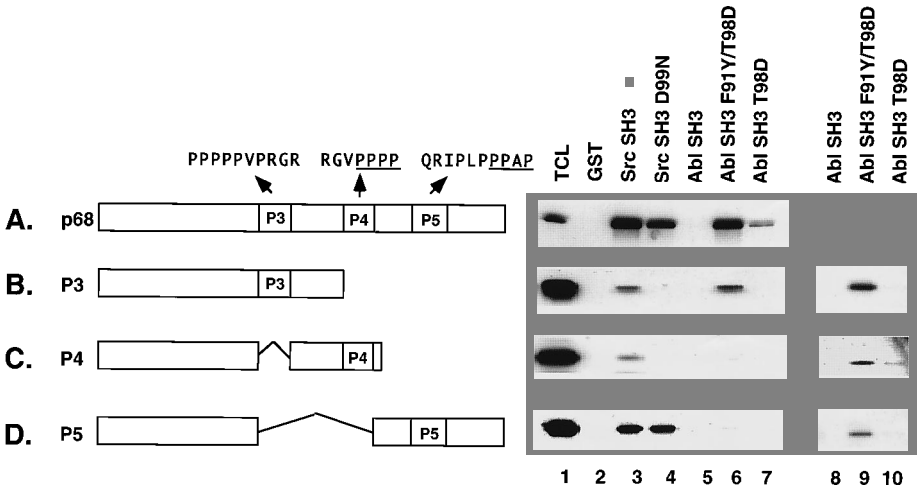


FIG. 5. Analysis of SH3 binding sites in p68 with the wild-type and mutant SH3 domains. Schematic diagrams representing wild-type p68-Myc and its deletion constructs used in the experiment are illustrated on the left. The amino acid sequences of three known SH3 binding sites, P3, P4, and P5, are also shown. Each plasmid construct was transfected into HeLa cells, and 100 μl of each Triton cell lysate was incubated with glutathione-agarose bound with GST (lane 2), GST-Src SH3 (lane 3), GST-Src SH3 D99N (lane 4), GST-Abl SH3 (lane 5 and longer-exposure lane 8), GST-Abl SH3 F91Y/T98D (lane 6 and longer-exposure lane 9), or GST-Abl SH3 T98D (lane 7 and longer-exposure lane 10). The bound proteins were eluted with SDS sample buffer, fractionated on a 10% polyacrylamide gel along with 5 μl of total cellular lysates (TCL) (lane 1), transferred to nitrocellulose, and probed with a mouse monoclonal antibody against Myc as described in Materials and Methods.

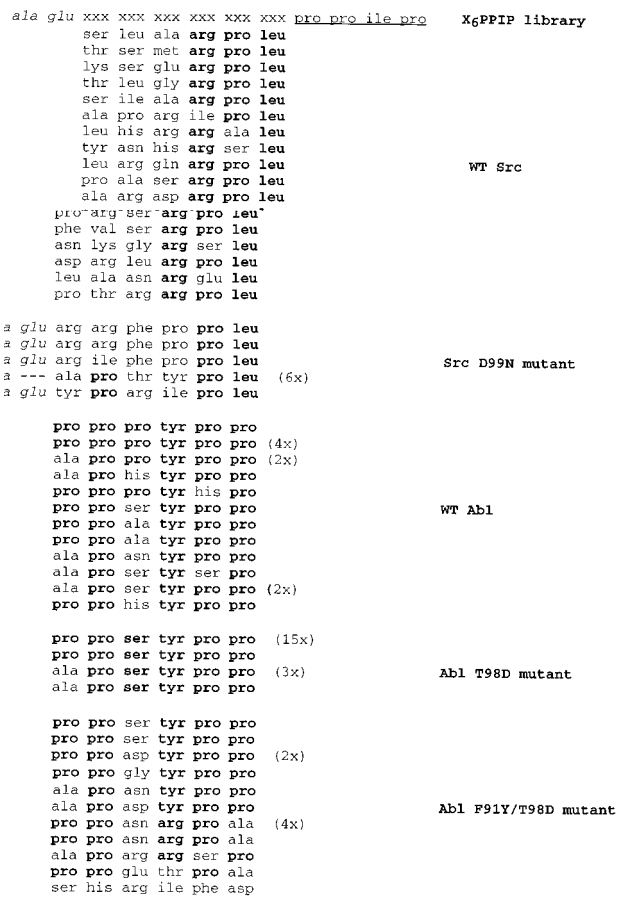
(Fig. 5A, lane 7) and more strongly to GST-Abl SH3 F91Y/T98D (Fig. 5A, lane 6). Although the wild-type Src SH3 could bind to p68-P3 (Fig. 5B, lane 3) as well as P68-P4 (Fig. 5C, lane 3), no apparent binding of the Src D99N mutant to either p68-P3 or p68-P4 was detected (lanes 4 in Fig. 5B and C), suggesting that binding of P3 and P4 to Src SH3 is dependent on Src Asp-99. Interestingly, the wild-type Src SH3 and the Src D99N mutant exhibited similar degrees of affinity to p68-P5 (Fig. 5D, lanes 3 and 4), suggesting that binding of P5 to Src SH3 is completely independent of Src Asp-99. Although GST-Abl SH3 did not bind to any of these p68 deletion variants (lanes 5 in Fig. 5B to D), GST-Abl SH3 F91Y/T98D could bind strongly to p68-P3 (Fig. 5B, lane 6 and longer-exposure lane 9) (the efficiency of the binding of this mutant to p68-P3 is similar to that of wild-type Src SH3 to p68-P3) and less strongly to p68-P4 (Fig. 5C, lane 6 and longer-exposure lane 9) and p68-P5 (Fig. 5D, lane 6 and longer-exposure lane 9). GST-Abl SH3 T98D, however, was found to only weakly bind to p68-P4 (Fig. 5C, lane 7 and longer-exposure lane 10).

Evaluation of the importance of an arginine-binding site on SH3 ligand selectivity by phage display libraries. We previously used phage display libraries to define amino acid sequences preferred by the SH3 domains of Src, Fyn, Lyn, PI3K, and Abl (17). Using the same technique, we assessed the importance of the Asp-99 in determining SH3 ligand selectivity. GST-Src SH3 D99N, GST-Abl SH3 T98D, and GST-Abl SH3 F91Y/T98D were each immobilized on polystyrene to select phages from a biased X_6PPIP library (consisting of six random amino acids upstream of the highly conserved PPIP motif). After several cycles of selection, the SH3-enriched phages were sequenced; the results are shown in comparison with previously published results for wild-type Src and Abl (Fig. 6).

Wild-type Src SH3 selected ligands with the consensus sequence XXXRPLPPIP from the X_6PPIP library. The D99N mutation did not affect selection at position −1 or −2 but completely eliminated selection of Arg at position −3. Instead, Pro, Tyr, or Ile was selected at this position. In addition, the D99N mutation appears to impose greater selectivity at positions −4, −5, and −6 than that detected for wild-type Src. All D99N-selected phage sequences, except the mutated sequence which lost the M13 gene III invariant glutamic acid residue, contained an Arg at position −4, −5, or −6.

Wild-type Abl SH3 showed a preference for the sequence PPPYPPPPIP, with Tyr selected at position −3 in 100% of phages examined. The Abl SH3 T98D mutant did not alter selection of Tyr at position −3 but did eliminate selection of Pro at position −4. This finding suggests that the T98D mutation alone was unable to confer binding selectivity for Arg at position −3, but rather changed the amino acid preference at the −4 position from a Pro to a Ser. However, one-third of the Abl SH3 F91Y/T98D mutant selected phage that displayed an Arg at position −3. This mutation did not eliminate the selection for Tyr at position −3, since at least one-half of the selected phage contained Tyr at this position. As with the T98D mutants, the selection for Pro at position −4 was lost in the double mutant. Therefore, the Abl SH3 F91Y/T98D mutant has dual sequence preferences: one resembles the wild-type Abl SH3 sequence recognition motif, and the other shows a similar specificity for Arg in the −3 position.

Determination of the affinity of wild-type and mutant Src and Abl SH3 for peptide ligands in a tryptophan fluorescence assay. To quantitatively evaluate the importance of Arg in the −3 position of SH3 peptide ligands, we measured the K_ds of the binding interactions between the SH3 domains used in this study and four defined synthetic peptides. These peptides varied at either position −3 or position −1 relative to the PXXP motif. For simplicity, the peptides were designated RPL (APA RPLPPLPGGK), RPP (APARPPPPLPGGK), YPP (APAYP PPPLPGGK), and YPL (APAYPLPPLPGGK). The variations were included at position −1 as well as position −3, since our previous phage selection data had shown that Src and Abl differ in preferences for amino acids at position −1 (Leu for Src and Pro for Abl) as well as position −3. Therefore, the amino acid in position −1 could influence the selection at position −3.



As shown in Table 1, Src SH3 showed a relatively high affinity for the RPL peptide (11.4 μM), and substitution of the

Previous nuclear magnetic resonance spectroscopy studies showed that the conserved tryptophans (for example, Trp-118 of Src in Fig. 1A) which comprise part of proline-binding pockets in an SH3 domain were perturbed upon ligand binding (27). In addition, structural analysis of the Src SH3-ligand complex has further demonstrated that Trp-118 of Src has extensive hydrophobic interactions with the ligand (Fig. 1A) (6). Since tryptophan fluorescence is extremely sensitive to the environment of the side chain, the ligand-induced fluorescence changes can be measured to calculate the K_d of a peptide by using Scatchard analysis (2). The K_d values of the four peptides are summarized in Table 1.

TABLE 1. Binding affinities of peptides

Peptide	Binding affinity (μM); mean ± SD)				
	Src	D99N	Abl	F91Y/T98D	T98D
RPL	11.4 ± 0.4	417 ± 121	UTD	UTD	UTD
RPP	UTD[a]	UTD	51 ± 2	59 ± 3	119 ± 6
YPL	89 ± 11	77 ± 7	UTD	UTD	UTD
YPP	UTD	UTD	6 ± 0.1	74 ± 16	95 ± 11

[a] UTD, unable to determine.

Arg with a Tyr caused an eightfold decrease in affinity (89 μM). Although the Src D99N mutation greatly decreased the affinity of Src SH3 for the RPL peptide by approximately 37-fold (417 μM), this mutant showed an affinity for the YPL peptide (77 μM) similar to that of wild-type Src SH3 (89 μM). Abl SH3, on the other hand, exhibited a high affinity for the YPP peptide (6 μM), and substitution of the Tyr with an Arg decreased the affinity of the peptide to Abl SH3 by roughly eightfold (51 μM). The T98D mutation in Abl SH3 decreased the affinity of this domain for the YPP peptide (74 μM) by 12-fold compared with wild-type Abl SH3 (6 μM). Interestingly, the T98D mutant exhibited similar degrees of affinity to the RPP and YPP peptides. Similar results were obtained with the Abl SH3 F91Y/T98D mutant. Addition of the RPL or YPL peptide to wild-type Src SH3 or the D99N mutant increased the fluorescence intensity around a wavelength of 320 nm, and Scatchard analysis of the data gave an accurate estimation of the K_d values as a result of good linear fitting. Similar results were obtained for wild-type Abl SH3 and its two mutants with the YPP and RPP peptides. However, addition of the RPP or YPP peptide to wild-type Src SH3 or the D99N mutant reduced the overall fluorescence intensity, and Scatchard analysis (linear fitting) of the fluorescence measurements had a poor correlation coefficient. Therefore, the K_d values could not be accurately calculated in these cases. Similar problems were found when the RPL and YPL peptides were used on Abl SH3 and its two mutants.

Evaluation of the affinities of the peptides to Src SH3 by competition assay. As mentioned above, the affinities of some of the peptides to the SH3 domains could not be accurately calculated because of the limitation of the tryptophan fluorescence assay. Therefore, we used a competition assay to examine the abilities of these four peptides to compete for proteins that interacted with Src SH3. Figure 7 shows an immunoblot from the competition assay probed with a monoclonal antibody against phosphotyrosine. In this assay, SRD 3T3 cell lysates were incubated with glutathione-agarose bound with GST (lane 1) and GST-Src SH3 (lanes 2 to 14) without peptide (lane 2) or with increasing amounts of either the RPL peptide (lanes 3 to 5), RPP peptide (lanes 6 to 8), YPL peptide (lanes 9 to 11), or YPP peptide (lanes 12 to 14). The RPL peptide blocked approximately 50% of binding of the 62-, 130-, 160, and 220-kDa tyrosine-phosphorylated proteins to Src SH3 at a concentration of 4 μM. Some degree of inhibition was also observed with YPL peptide at 64 μM. However, no apparent inhibition was observed with the RPP or YPP peptide even at 64 μM, suggesting that these two peptides have even a lower affinity for Src SH3 than the YPL peptide does.

DISCUSSION

SH3 specificity for cellular binding proteins determined by the aspartic acid in the arginine binding site. In this study, we examined the importance of the Asp-99 residue of Src in determining SH3 ligand specificity by analyzing the binding properties of a mutant Src SH3 domain containing a Asn substitution for Asp-99. In addition, we examined whether substituting Thr-98 with an Asp residue in the analogous binding site of Abl SH3 would alter the binding specificity of this domain, possibly conferring selectivity for Arg in the −3 position of peptide ligands.

Using an affinity binding assay, we showed that binding of the Src SH3 domain to most cellular proteins (including hnRNP K) is dependent on Asp-99; however, binding to certain proteins (such as a v-Src substrate, p68) was not significantly affected by the D99N substitution. These results indicate that an Asp residue at position 99 of Src SH3 plays a dominant role in the selection of cellular binding proteins; however additional or alternate contact sites within the SH3 domain must be involved in the selection of other Src SH3-binding proteins.

In an attempt to understand the structural basis for these two different binding mechanisms, we compared the SH3 binding sites in hnRNP K and p68. In hnRNP K, the SH3 binding site has been mapped to a 36-amino-acid region containing two proline-rich stretches (RGPPPPP and RNLPLPP) (24). The presence of an Arg_{-3} in both motifs can explain the strong dependence on Asp-99 for hnRNP K binding. On the other hand, p68 contains three SH3 binding sites: P3 (PPPPPVPR GR), P4 (RGVPPPP), and P5 (PLPPPAP). Src SH3 bound with similar efficiencies to the P4 site (with an N-terminal Arg_{-3} that is similar to a class I SH3 ligand) and P3 site (with two C-terminal arginines similar to class II ligands), yet showed better recognition of the P5 site (which lacks an Arg). We thus hypothesized that binding of the arginine-containing sites (P3 and P4) to the Src SH3 domain would be dependent on Src Asp-99, whereas binding of P5, which lacks an arginine, would be independent of Src Asp-99. The results from the affinity binding assay using the p68 deletion constructs containing either P3, P4, or P5 supported this hypothesis (Fig. 5).

It is worth noting that although p68-P3 has two C-terminal arginines, neither of the two arginine residues is in the position of the arginine in consensus class II Src SH3 ligands (6). Interestingly, previous combinatorial peptide library screening with Src SH3 also revealed a ligand (MMAPPLPRL) with an

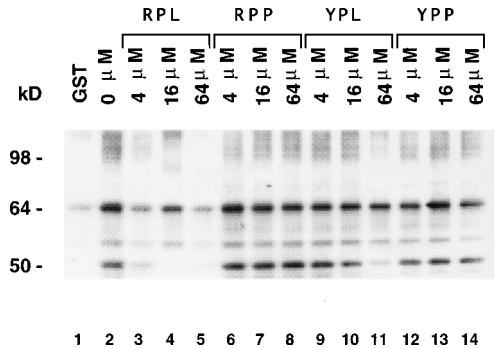


FIG. 7. Competition assay. SRD 3T3 cell RIPA lysates were incubated with glutathione-agarose bound with GST (lane 1) or GST-Src SH3 (lanes 2 to 14) without peptide (lane 2) or with increasing amounts of the peptides RPL (lanes 3 to 5), RPP (lanes 6 to 8), YPP (lanes 9 to 11), and YPL (lanes 12 to 14). The final concentrations of the peptides used in the experiment are indicated. The bound proteins were eluted with SDS sample buffer, fractionated on a 10% polyacrylamide gel, transferred to nitrocellulose, and probed with the phosphotyrosine monoclonal antibody 4G10 as described in Materials and Methods.

Arg in a position different from that of the Arg in the consensus sequence (26):

class II ligand consensus	XPPXPXR
p68-P3	PPPVPRGR
a ligand from peptide library (26)	MMAPPLPRL

Since binding of p68-P3 is dependent on Asp-99 of Src, we speculate that the Arg residue(s) in p68-P3 might still be able to interact with Asp-99 of Src even though it is not in the expected position.

It is somewhat puzzling that the P5 binding site, which exhibited efficient SH3 binding, lacks an Arg, since Arg has been clearly shown to be important for high-affinity binding of SH3 peptide ligands to Src SH3 (for example, we found that the RPL peptide has an eightfold-higher affinity for the Src SH3 domain than the YPL peptide does [Table 1]). One possible explanation is suggested by recent findings from phage display indicating that the residues flanking the core Src SH3 binding motif RPLPPLP can contribute significantly to the affinity of a ligand for the SH3 domain (18). Hence, the flanking sequence interactions could compensate for the absence of the Arg-Asp interaction involved in the p68-P5 fragment binding to Src SH3.

The importance of the arginine binding site in determining SH3 specificity has been further highlighted by the affinity binding assay showing that the T98D substitution enabled Abl SH3 to weakly bind to both hnRNP K and p68. We also showed that the binding of p68 to the Abl SH3 T98D mutant is mediated mainly through P4, which contains an Arg_{-3} perhaps through the interaction between the artificially introduced Asp-98 in the Abl SH3 domain and Arg_{-3} in the p68-P4 (RGVPPPP). Interestingly, the double mutant F91Y/T98D exhibited a higher affinity for both the wild-type p68 and the deletion mutants than the T98D mutant did. Most remarkably, this double mutant showed binding efficiency for the p68-P3 mutant similar to that of wild-type Src SH3. Since it has been shown that Tyr-92 and Asp-99 form a hydrogen bond in Src SH3, a similar hydrogen bond may exist in the Abl SH3 double mutant, which may enhance the affinity of the domain for certain ligands.

Taken together, our results shown that Src SH3 binds to cellular proteins by two mechanisms, one involving Asp-99 and the other independent of Asp-99. Furthermore, creating an artificial arginine binding site in Abl SH3 can modify the Abl SH3 binding specificity to resemble that of Src SH3. These results support the hypothesis that conserved amino acids from the SH3 binding site determine the core binding sites, whereas nonconserved residues confer the binding specificity for each SH3 domain (6, 11, 26, 27).

Recently Erpel and coworkers (5) have presented evidence that a lysine substitution of Asp-99 leads to a deregulation of Src activity, suggesting that Asp-99 may be involved in intramolecular interactions that negatively regulate Src activity. However, this Lys substitution could potentially affect the overall folding of the SH3 domain.

SH3 peptide ligand preference. The importance of Asp-99 in the selection of an Arg_{-3} in the Src SH3 ligands was further strengthened by the results from the phage display libraries. Unlike wild-type Src SH3, which selected the sequence RPL in the three amino acids upstream from the invariant PPIP in the biased library, the Src SH3 D99N mutant selected the sequence (P/Y/I)PL at these positions. In addition, this mutation appeared to impose the selection of an Arg in positions further upstream. The Arg residues selected at the −6 and −5 positions might interact with acidic residues other than Asp-99 in Src SH3, thus compensating for absence of the Asp-99 binding interactions. Interestingly, although the Asp-99-independent P5 site of p68 does not contain an Arg at the −3 position, it contains an Arg at the −5 position which could be involved in alternate binding interactions similar to those utilized by the D99N mutant domain.

Using a tryptophan fluorescence assay, we quantitatively compared the affinities of wild-type and mutant Src SH3 and Abl SH3 for a defined set of four peptide ligands which varied in the amino acids at the −3 and −1 positions upstream of the PXXP motif (Table 1). We found that Src SH3 prefers an Arg_{-3}, since substitution of the Arg with a Tyr caused an eightfold decrease in affinity. The preference of an Arg_{-3} can be attributed to Asp-99, since the D99N mutation lowered the affinity of the RPL peptide for Src SH3 by 37-fold. Abl SH3, on the other hand, prefers a Tyr_{-3}, since substitution of the Tyr with an Arg also lowered the peptide affinity by eightfold. Abl Thr-98 appears to be important for this preference, since the T98D mutation lowered the affinity of Abl SH3 for the YPP peptide by 12-fold.

It is interesting that although the Src SH3 D99N mutant has a 37-fold-lower affinity for the RPL peptide than wild-type Src SH3 does, the D99N mutant and wild-type Src SH3 showed similar affinities for the YPL peptide. In addition, the mutant and wild-type Src SH3 bound equally well to p68-P5 (Fig. 5), suggesting that the decrease in the affinity of the D99N mutant for the RPL peptide and the lack of binding of most cellular proteins to this mutant is not due to a dramatic alteration or instability of the protein structure caused by the mutation as previously suspected (10) but rather is due to a modification of binding specificity.

Because of the limitation of the tryptophan fluorescence assay, we could not accurately measure the affinity of the RPP or YPP peptide for Src SH3. However, the peptide competition assay has shown that the RPP and YPL peptides did not exhibit any degree of inhibition at 64 μM, while the YPP peptide showed detectable inhibition at this concentration (Fig. 7). Combining the results from the peptide competition and tryptophan fluorescence assay, we can conclude that Src SH3 has the highest affinity for the RPL peptide, an eightfold-lower affinity for the YPL peptide, and an even lower affinity for the RPP and YPP peptides. Thus, Src SH3 appears to prefer Leu_{-1}-Pro_0 to Pro_{-1}-Pro_0 following the Arg_{-3} at least in the context of this peptide. On the other hand, Abl SH3 seems to prefer Pro_{-1}-Pro_0 to Leu_{-1}-Pro_0 following the Tyr_{-3}. Although the structural basis for this preference is not clear, overlaying the three-dimensional structures of Src SH3 and Abl SH3 reveals that Abl SH3 has a slightly wider binding site (the second binding pocket formed by Tyr-92, Trp-118, Pro-133, and Tyr-136) than Src SH3. Therefore, the Pro-Pro dipeptide may be geometrically more demanding for Src SH3 than Leu-Pro, while the larger binding pocket in Abl SH3 may be ideal for accommodating Pro-Pro.

Two lines of evidence suggest that at least the F91Y/T98D substitution can partially confer arginine binding specificity to Abl SH3. First, F91Y/T98D recognized PPPPPVPRGR and RGVPPPP peptides in the context of p68-P3 and p68-P4. Second, the Abl SH3 F91Y/T98D mutant selected two types of ligands from phage display libraries: one contained a Tyr_{-3} (as in wild-type Abl SH3) and the other contained an Arg_{-3}. In tryptophan fluorescence assays using peptide ligands, the T98D and F91Y/T98D mutants exhibited similar affinities for the YPP and RPP peptides, consistent with the F91Y/T98D mutant selection of Tyr or Arg in the −3 position of ligands from the phage library. Thus, the mutation of T98D/F91Y did not eliminate selection of Tyr at the −3 position but did confer

recognition of an Arg at this site. It is likely that the selections at position −3 are influenced by the amino acids flanking this position.

These studies demonstrate the importance of an acidic residue within the third binding site of SH3 domains in ligand selection and also show that such an acidic residue is not the only structural determinant involved in ligand selection at this site.

Change of nomenclature. The Src SH3-associated p62 reported in our previous paper (24) has been renamed p68 in this paper since it has an apparent molecular mass of 68 kDa and does not constitute the major GTPase-activating protein-associated 62-kDa tyrosine-phosphorylated protein, as suggested by our unpublished results as well as the results from other groups (7, 12, 22, 25).

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REFERENCES

1. **Cheadle, C., Y. Ivashchenko, V. South, G. H. Searfoss, S. French, R. Howk, G. A. Ricca, and M. Jaye.** 1994. Identification of a Src SH3 domain binding motif by screening a random phage display library. J. Biol. Chem. **269:**24034–24039.
2. **Chen, J. K., W. S. Lane, A. W. Brauer, A. Tanaka, and S. L. Schreiber.** 1993. Biased combinatorial libraries: novel ligands for the SH3 domain of phosphatidylinositol 3-kinase. J. Am. Chem. Soc. **115:**12591–12592.
3. **Cicchetti, P., B. J. Mayer, G. Thiel, and D. Baltimore.** 1992. Identification of a protein that binds to the SH3 region of Abl and is similar to Bcr and GAP-rho. Science **257:**803–806.
4. **Cohen, G. B., R. Ren, and D. Baltimore.** 1995. Modular binding domains in signal transduction proteins. Cell **80:**237–248.
5. **Erpel, T., G. Superti-Furga, and S. A. Courtneidge.** 1995. Mutational analysis of the Src SH3 domain: the same residues of the ligand binding surface are important for intra- and intermolecular interactions. EMBO J. **14:**963–975.
6. **Feng, S., J. K. Chen, H. Yu, J. A. Simon, and S. L. Schreiber.** 1994. Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. Science **266:**1241–1247.
7. **Fumagalli, S., N. F. Totty, J. J. Hsuan, and S. A. Courtneidge.** 1994. A target for Src in mitosis. Nature (London) **368:**871–874.
8. **Matunis, M. J., W. M. Michael, and G. Dreyfuss.** 1992. Characterization and primary structure of the poly(C)-binding heterogeneous nuclear ribonucleoprotein complex K protein. Mol. Cell. Biol. **12:**164–171.
9. **Mayer, B. J., and D. Baltimore.** 1993. Signalling through SH2 and SH3 domains. Trends Cell. Biol. **3:**8–13.
10. **Musacchio, A., M. Saraste, and M. Wilmanns.** 1994. High-resolution crystal structures of tyrosine kinase SH3 domains complexed with proline-rich peptides. Nat. Struct. Biol. **1:**546–551.
11. **Noble, M. E. M., A. Musacchio, M. Saraste, S. A. Courtneidge, and R. K. Wierenga.** 1993. Crystal structure of the SH3 domain in human Fyn; comparison of the three-dimensional structures of SH3 domains in tyrosine kinases and spectrin. EMBO J. **12:**2617–2624.
12. **Ogawa, W., Y. Hosomi, K. Shii, and R. A. Roth.** 1994. Evidence for two distinct 60-kilodalton substrates of the SRC tyrosine kinase. J. Biol. Chem. **269:**29602–29608.
13. **Pawson, T.** 1988. Non-catalytic domains of cytoplasmic protein-tyrosine kinases: regulatory elements in signal transduction. Oncogene **3:**491–495.
14. **Pawson, T., and G. Gish.** 1992. SH2 and SH3 domains: from structure to function. Cell **71:**359–362.
15. **Ren, R., B. J. Mayer, P. Cicchetti, and D. Baltimore.** 1993. Identification of a ten-amino acid proline-rich SH3 binding site. Science **259:**1157–1161.
16. **Richard, S., D. Yu, K. Blumer, D. Hausladen, M. W. Olszowy, P. A. Connelly, and A. S. Shaw.** 1995. Association of p62, a multiple SH2- and SH3-domain-binding protein, with Src family tyrosine kinases, Grb2, and phospholipase Cγ-1. Mol. Cell. Biol. **15:**186–197.
17. **Rickles, R. J., M. C. Botfield, Z. Weng, J. A. Taylor, O. M. Green, J. S. Brugge, and M. J. Zoller.** 1994. Identification of Src, Fyn, Lyn, PI3K and Abl SH3 domain ligands using phage display libraries. EMBO J. **13:**5598–5604.
18. **Rickles, R. J., M. C. Botfield, X.-M. Zhou, P. A. Henry, J. S. Brugge, and M. Zoller.** Phage display selection of ligand residues important for SH3 domain binding specificity. Proc. Natl. Acad. Sci. USA, in press.
19. **Siomi, H., M. C. Siomi, R. L. Nussbaum, and G. Dreyfuss.** 1993. The protein product of the fragile X gene, FMR1, has characteristics of an RNA-binding protein. Cell **74:**291–298.
20. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. Gene **67:**31–40.
21. **Sparks, A., L. A. Quilliam, J. M. Thorn, C. J. Der, and B. K. Kay.** 1994. Identification and characterization of Src SH3 ligands from phage-displayed random peptide libraries. J. Biol. Chem. **269:**23853–23856.
22. **Taylor, S. J., and D. Shalloway.** 1994. An RNA-binding protein associated with Src through its SH2 and SH3 domains in mitosis. Nature (London) **368:**867–870.
23. **Weng, Z., J. A. Taylor, C. E. Turner, J. S. Brugge, and C. Seidel-Dugan.** 1993. Detection of Src homology 3-binding proteins, including paxillin, in normal and v-Src-transformed Balb/c 3T3 cells. J. Biol. Chem. **268:**14956–14963.
24. **Weng, Z., S. M. Thomas, R. J. Rickles, J. A. Taylor, A. W. Brauer, C. Seidel-Dugan, W. M. Michael, G. D. Dreyfuss, and J. S. Brugge.** 1994. Identification of Src, Fyn, and Lyn SH3-binding proteins: implications for a function of SH3 domains. Mol. Cell. Biol. **14:**4509–4521.
25. **Wong, G., O. Muller, R. Clark, L. Conroy, M. F. Moran, P. Polakis, and F. McCormic.** 1992. Molecular cloning and nucleic acid binding properties of the GAP-associated tyrosine phosphoprotein p62. Cell **69:**551–558.
26. **Yu, H., J. K. Chen, S. Feng, D. C. Dalgarno, A. W. Brauer, and S. L. Schreiber.** 1994. Structural basis for the binding of proline-rich peptides to SH3 domains. Cell **76:**933–945.
27. **Yu, H., M. K. Rosen, T. B. Shin, C. Seidel-Dugan, J. S. Brugge, and S. L. Schreiber.** 1992. Solution structure of the SH3 domain of Src and identification of its ligand-binding site. Science **258:**1665–1668.